Acute Actions of Alcohol on the Brain

Unlike many drugs that have a single site of action, alcohol produces a wide spectrum of effects on brain function via interaction with multiple targets. For many years, it was assumed that alcohol's effects on brain function were primarily due to its ability to nonspecifically disrupt or disorder the neuronal membrane. This hypothesis, which originated nearly 100 years ago, has been severely challenged by research carried out over the last 10 years.

A growing body of evidence shows that alcohol exerts significant effects on specific receptor proteins that are embedded in the neuronal plasma membrane. The function of these proteins ultimately underlies all brain function, including thought, speech, vision, and complex behaviors. This view, which is becoming widely accepted, represents a dramatic change in the way we think about alcohol and its effects on brain activity. The factors that precipitated this new way of thinking about alcohol are closely tied to the advances made in the field of molecular neuroscience and to technical advances made in the way that the activity of neurons is studied.

Because of the complexity of the human brain, which is comprised of approximately 1 trillion neurons, scientists have developed techniques to study brain function in simpler systems where there are fewer experimental variables. These techniques include brain cell cultures that permit the behavior of single neurons to be studied, and the use of recombinant or cloned receptors that can be expressed in experimental cells such as frog eggs (*Xenopus* oocytes). Experiments using microdialysis, which allows the fluid that surrounds neurons to be repeatedly sampled, have allowed researchers to examine the effects of alcohol on levels of neurotransmitters in intact animals. Finally, behavioral approaches have allowed scientists to gain important insights into the effects of alcohol on brain function in awake, freely moving animals.

These approaches have resulted in a remarkably detailed understanding of the effects of acute alcohol exposure on many important neuronal proteins that regulate brain activity. By understanding the molecular and cellular sites of action for the acute effects of alcohol, it should be possible to explain how the continued use of alcohol may lead to alcohol dependence or how the abuse of alcohol may result in death or serious brain injury.

The following material is not intended to be an exhaustive literature review. Rather, it is a focused examination of areas that have seen significant progress in the last several years.

Measuring Alcohol's Effects

As with many drugs, alcohol's effects are dose dependent. That is, higher concentrations of alcohol generally produce a larger effect on a particular protein or biological process, or a more marked effect on behavior or function, than lower concentrations do. Because alcohol is administered by drinking and is distributed to the brain by the bloodstream, alcohol concentrations are usually measured in terms of concentration, or percent, in the blood. In most states, for example, individuals with blood alcohol levels (BAL's) or blood alcohol concentrations (BAC's) of 0.1 percent or higher are considered legally intoxicated. (See the box "The ABC's of BAC's" in the chapter on prevention research for additional background.)

Most researchers who conduct alcohol research using in vitro (cellular or test tube) preparations prefer to express concentrations of alcohol as molar concentrations (the amount of alcohol in solution in a given volume of water based on the number of molecules, or moles, of alcohol present). For example, a 0.1-percent BAL is the same as 22.5 millimolar (mM). In these types of experimental research studies, concentrations of

alcohol that are considered relevant with respect to their behavioral effects are generally in the range of 10 to 100 mM. These levels are considered biologically important because it is possible to show significant effects of alcohol on some neuronal proteins, pathways, or systems simply by increasing the alcohol concentration. However, to be considered a relevant target for alcohol's actions, a protein or process should be sensitive to concentrations of alcohol that are associated with changes in human behavior and are easily achieved during alcohol drinking.

Alcohol and Ion Channels

Considerable evidence has accumulated to show that alcohol exerts significant effects on specific receptor proteins that are embedded in the neuronal plasma membrane. Of particular interest are reports that have demonstrated that alcohol alters the function of several important voltage-gated and ligand-gated ion channels. These channels are located predominantly in the synapse, where they mediate and modulate neuronal excitability. Since alcohol's effects are most noticeable at the synapse, these ion channels represent excellent targets for alcohol's anesthetic and intoxicating actions. Recent studies show that alcohol exerts powerful and specific effects on ion channel function and that these effects may be modulated by the subunit makeup and state of phosphorylation of these ion channels (for background on terms used in this discussion see the section "Setting the Stage: The Structure and Function of Neurons" earlier in this chapter).

Inhibitory Ligand-Gated Ion Channels

Research shows that many compounds that produce sedation and anesthesia appear to operate by enhancing the effects of gamma-aminobutyric acid (GABA) on the $GABA_A$ receptor. Among these compounds is the large class of sedative-hypnotic drugs that include benzodiazepines (Valium), barbiturates (pentobarbital), general anesthetics (halothane), and alcohol.

Endogenous (naturally present in an organism) substances also enhance $GABA_A$ receptor function and thus help regulate the activity of the receptor. The cloning of members of the $GABA_A$ family of subunits has revealed that neurons express multiple types of $GABA_A$ receptors that are made up of different subunits, as discussed in the section "From Cell Membrane to Nucleus: The Effects of Alcohol on Brain Neurons" earlier in this chapter. The combination of different receptor subunits yields receptors with different sensitivities to both endogenous compounds such as GABA and exogenous drugs such as alcohol.

GABA Receptors.

Alcohol Potentiation of $GABA_A$ Receptor Function. How alcohol potentiates the effect of GABA on its receptors is not clear. It is likely that the sensitivity of $GABA_A$ receptor to alcohol is complex and subject to multiple levels of regulation. One approach to studying the complexities of the $GABA_A$ receptor subunit composition uses a technique known as recombinant complementary DNA (cDNA) expression systems, in which part of the genetic material (deoxyribonucleic acid, or DNA) from one organism is inserted into the genetic material of a second organism, such as bacteria or *Xenopus* oocytes. Such systems allow investigators to express, or produce, individual or multiple $GABA_A$ receptor subunits in experimental cells that normally do not express these receptors. This technique allows for systematic evaluation of the effects of alcohol on different receptor subunit combinations. This approach has proven valuable in showing that some $GABA_A$ receptor responses in neurons are sensitive to alcohol, whereas others are not. This implies that the subunit makeup of the $GABA_A$ receptor may be an important determinant of sensitivity to alcohol's actions. If true, then differences in the $GABA_A$ receptor subunit composition could underlie the observed differences in alcohol sensitivity between alcoholics and nonalcoholics.

Research using recombinant cDNA expression systems has suggested that the γ_{2L} subunit of the $GABA_A$ receptor is critical to alcohol's potentiating effect (Wafford et al. 1991). Not all such studies have demonstrated a potentiating effect,

however, even in the presence of the γ_{2L} subunit (Sigel et al. 1993). Thus, although this subunit may be necessary for alcohol to have an effect, other factors may be important in determining whether such an effect is observed. These other factors may include phosphorylation (the addition of a phosphate group through enzymes known as protein kinases), which has been shown to influence the regulation of receptor activity; association of the $GABA_A$ subunits with the cellular cytoskeleton (the structural architecture of the cell); and location of the receptor on the cell membrane.

For example, one recent study added to earlier evidence that the phosphorylating enzyme protein kinase C (PKC) is involved in modulating alcohol sensitivity of GABA receptors (Weiner et al. 1997*a*). In this study of brain slices from adult rats, basal (before alcohol treatment) levels of PKC activity correlated with alcohol's potentiation of $GABA_A$ receptor responses. (By varying incubation temperatures of the brain slices, the investigators altered basal PKC activity levels.) Treatment of neurons with drugs that reduced basal PKC activity also reduced the effects of alcohol. Activation of other kinases, such as protein kinase A, also has been shown to enhance the sensitivity of $GABA_A$ receptors to alcohol (Freund and Palmer 1997*a*; Lin et al. 1994). Data from these studies suggest that the state of receptor phosphorylation is important in determining the sensitivity of $GABA_A$ receptors to alcohol. Direct measures of receptor phosphorylation are needed to confirm this suggestion.

Another aspect of ion channel function is emerging from current, ongoing neuroscience research. Recent studies suggest that the neuron is not an unorganized collection of proteins bounded by the lipid membrane, but a highly structured, three-dimensional environment in which the function of a receptor or ion channel is influenced by its location. This spatial organization may be important in clustering together proteins that interact with one another, such as kinases and ion channels like the $GABA_A$ receptor. A recent report illustrated the importance of this clustering to the effects of alcohol (Whatley et al. 1996). In this study, $GABA_A$ receptor subunits were introduced into a cell line that did not normally express these receptors. Alcohol potentiated the GABA responses in these cells; however, agents that affected the cytoskeleton of the cell prevented this potentiation. The disruption of the cytoskeleton may have prevented the $GABA_A$ subunits from being phosphorylated by protein kinases because of a physical separation of the receptor and kinase.

Reinforcing the idea that receptor localization can influence alcohol sensitivity, a study using rat brain slices showed that alcohol potentiated $GABA_A$ receptors located in the synapses on some dendrites of a single neuron but not in others (Weiner et al. 1997*b*). The underlying reason for this striking effect was not elucidated. Nevertheless, taken together, the findings described above suggest that factors other than $GABA_A$ receptor subunit makeup help determine the sensitivity of the brain to alcohol. They also raise the possibility that psychological or pharmacologic interventions that alter neuronal excitability may alter an individual's response to alcohol.

Effects of Alcohol on $GABA_A$ Receptor Knockout Animals. Despite evidence from in vitro studies that $GABA_A$ receptor subunit makeup does not appear to be the sole determinant of alcohol's actions, it is not clear whether this is true in intact animals. One direct approach to testing how specific receptor subunits mediate alcohol's effects is to develop genetically altered animals that are lacking functional specific receptor subunits. These knockout animals have become a valuable tool to examine the involvement of specific receptors in mediating the actions of alcohol on the brain. (Two other sections in this chapter, "From Cell Membrane to Nucleus: The Effects of Alcohol on Brain Neurons" and "Genetic Studies of Alcohol's Actions on the Brain," also discuss knockout animals.)

To date, alcohol sensitivity has been determined in animals lacking the $GABA_A$ receptor subunits

alpha (α), beta (β), and gamma (γ). Studies of mice that lack the α_6 subunit of the $GABA_A$ receptor have shown, for example, that the α_6 subunit does not mediate the sedative-hypnotic actions of alcohol and other depressant agents, as measured by alcohol-induced hypnosis (sleep time) (Homanics et al. 1997). Whether this subunit mediates other actions of alcohol that may occur at nonsedative concentrations remains to be determined.

In another study, scientists compared the anesthetic sensitivity of knockout mice lacking the β_3 subunit of the $GABA_A$ receptor with that of control (or wild-type) mice in which the functional gene is present (Quinlan et al. 1998). Knockout mice showed no significant differences in the sedative effects of alcohol, pentobarbital, or volatile anesthetics (enflurane or halothane) compared with control mice. However, the knockout mice were more resistant to the surgical anesthetic (loss of sensation) effects of the volatile anesthetics. These data indicate that the β_3 subunit does not play a critical role in the sedative effects of alcohol. These findings also suggest the presence of different sites of action for the sedative and anesthetic actions of commonly used surgical anesthetic agents.

Another subunit, the γ_{2L} subunit already mentioned, previously had been shown by some investigators to be important in mediating the potentiating effects of alcohol on $GABA_A$ receptor function (Wafford et al. 1991). (Again, not all such studies have demonstrated a potentiating effect.) If the γ_{2L} subunit is required for this action in intact animals, animals lacking this subunit would be expected to show an altered response to alcohol. Two studies have sought to test this hypothesis by examining the effects of knocking out the gene that codes for the γ_2 subunit of the $GABA_A$ receptor.

A recent study examined the alcohol sensitivity of mice lacking only the γ_{2L} subunit of the $GABA_A$ receptor (Homanics et al. in press). The animals lacking only the γ_{2L} variant of the $GABA_A$ receptor had normal life spans (most mice lacking the entire γ_2 gene die within a few days of birth [Gunther et al. 1995]) and were not significantly different from wild-type mice in their behavioral responses to alcohol, including sleep time, anxiolysis (anxiety reduction), acute functional tolerance, withdrawal hyperexcitability (over-excitability that occurs after exposure to, then removal of, alcohol), and hyperlocomotion (Homanics et al. in press). In addition, in the presence of alcohol, GABA-induced electrical activity in neurons isolated from these knockout mice was stimulated to the same extent as neurons isolated from wild-type mice. Several behavioral effects of alcohol remained unchanged in the knockout mice. These results suggest that the γ_{2L} subunit is not essential for alcohol effects on the $GABA_A$ receptor.

As noted in the section "From Cell Membrane to Nucleus: The Effects of Alcohol on Brain Neurons" earlier in this chapter, scientists have also examined the alcohol sensitivity of mice lacking the γ isoform of the phosphorylating enzyme PKC (PKC-γ). Mutant mice lacking PKC-γ showed a reduced sensitivity to both the sedative and hypothermic effects of alcohol (Harris et al. 1995). In addition, the ability of alcohol to potentiate $GABA_A$ receptor function in brain tissue from these mice was reduced compared with that in control animals. Interestingly, the knockout mice had normal responses to other sedatives such as benzodiazepines and barbiturates that are thought to interact with the $GABA_A$ receptor. Taken with the results of the research described above, these data support the idea that receptor phosphorylation and other posttranslational processes (processes that take place after the last step in gene-directed protein synthesis) may be more important than $GABA_A$ receptor subunit makeup in determining the alcohol sensitivity of these receptors.

Glycine Receptors. Both GABA receptors and glycine receptors—to which the inhibitory neurotransmitter glycine binds—mediate the flux of chloride ions into neurons; this process, in turn, inhibits the cell's ability to fire, or transmit, a nerve impulse. Glycine receptors are found predominantly in the spinal cord, but they also exist in the brains, where they may play a role

in some of the actions of alcohol. As demonstrated using $GABA_A$ receptors, alcohol also potentiates chloride flux—augments the entry of negatively charged chloride ions into the neuron—through recombinant glycine receptors expressed in *Xenopus* oocytes (Mascia et al. 1996). Glycine receptors composed of the α_1 subunit were more sensitive to low concentrations of alcohol than those made from α_2 subunits. This difference was attributed to a change in a single amino acid of the α_2 subunit, suggesting that alcohol may interact with specific amino acids on this ion channel to cause its effects.

This hypothesis is supported further by data suggesting that the potentiation of recombinant glycine and $GABA_A$ receptors by all concentrations of alcohol could be abolished by single-amino acid substitutions in either the GABA transmembrane II (TMII) or TMIII domains of the neuronal cell membrane (Mihic et al. 1997). For example, when the amino acid serine at position 267 in the TMII domain of the glycine α_1 receptor was changed to the amino acid isoleucine, electrical currents generated by this mutant receptor were no longer potentiated by alcohol. Changing the amino acid serine at position 267 to another amino acid, tyrosine, abolished the effects of some but not all anesthetic agents that share some behavioral effects with alcohol. Subsequent work demonstrated that alcohol's effect on recombinant glycine α_1 receptors was inversely correlated with the size of the amino acid at position 267 (Ye et al. 1998). Thus, alcohol potentiated receptor function when small amino acids such as glycine or alanine were substituted at that position. However, substituting large amino acids such as histidine, cysteine, or tyrosine resulted in receptors that were inhibited by alcohol.

These findings provide strong evidence that alcohol interacts with discrete regions of important neuronal proteins to alter their function. Furthermore, alcohol may preferentially partition into pockets defined by specific amino acids in areas of the receptor protein that are important for cell functioning. Alcohol's presence in these pockets may disrupt the normal interactions between various regions of the receptor, leading to altered receptor function. This research also raises the possibility that changes in specific amino acids brought about by either experimental or random changes to an individual's genetic code may lower an individual's sensitivity to the sedative and anesthetic effects of alcohol that appear to be mediated via glycine and $GABA_A$ receptors. Development of experimental animals with glycine receptors possessing specific amino acid substitutions will allow researchers to test these hypotheses.

Excitatory Ligand-Gated Ion Channels

Glutamate Receptors. As the major excitatory amino acid in the brain, glutamate binds to specific ligand-gated ion channels and depolarizes the postsynaptic neuronal membrane, making it more likely that the neuron will "fire." In this way, these proteins serve as excitatory ion channels. Three distinct families of glutamate-activated ion channels are present on the postsynaptic, dendritic membrane of neurons. These receptors are named for compounds that selectively activate one class of receptors but not the others. For example, NMDA receptors are named for the synthetic compound *N*-methyl-D-aspartate (NMDA), whereas AMPA receptors are activated by the synthetic compound alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA). The natural chemical kainate activates a subset of ligand-gated ion channels that are similar in structure to the AMPA family and that are sometimes grouped together in a classification termed AMPA/kainate receptors. Distinct genes for these three classes of glutamate-activated ion channels have now been cloned, allowing researchers to study the effects of alcohol on specific receptor combinations.

NMDA receptors are categorized by constituent subunits: the NR1 subunits and four members of the NR2 family (A, B, C, and D). AMPA/kainate receptors are represented by at least seven subunit types (GluR1 through GluR7) plus two other subunits (KA1 and KA2) that respond selectively to kainate. Slight differences in the amino acid sequence of certain members of each of these families create an additional level of

receptor diversity. For example, there are eight different versions of the NR1 subunit that differ by the absence or presence of three sequences of amino acids.

As a group, the different glutamate receptor subunits are distributed widely throughout the brain, although certain subunits are found in very limited areas. Receptors composed of different subunits show slightly different sensitivities to activation by glutamate; these sensitivities are often manifested in the amount of time that the receptors remain open after being activated. Glutamate receptors also differ in the selectivity of the ions that permeate their pore.

AMPA/kainate receptors are permeable largely to sodium ions and, when activated by glutamate, set off a rapid and large depolarization of the postsynaptic neuron. If enough of these receptors are activated, the cell can fire an action potential, and neuronal signaling proceeds to the next neuron. NMDA receptors allow both sodium and calcium ions to enter the cell. Unlike AMPA/kainate receptors, however, NMDA receptors are blocked by magnesium ions, which are normal constituents of the extracellular fluid that bathes neurons. This block is voltage dependent, meaning that under conditions of relatively low-frequency electrical activity, NMDA receptors are blocked by magnesium ions, allowing few calcium ions to enter the neuron. When more glutamate is released into the synapse, sodium flowing through AMPA/kainate receptors depolarizes the neuron so that the magnesium block of the NMDA receptor is removed, calcium ions can enter the neuron, and the neuron's ability to fire a nerve impulse increases.

Calcium is an important regulator of neuronal function that exerts its effects through its interactions with a wide variety of enzymes and other proteins. For example, substantial evidence suggests that the entrance of calcium ions into the neuron through the NMDA receptor is required for the formation of new memories, such as those that take place during learning. NMDA receptors are also important during the period of development when brain neurons organize themselves into the complex circuits that allow the brain to support people's ability to speak, think, reason, and respond to others. In humans, the period of most intense growth and development of these circuits begins during the last stages of fetal development and accelerates to a peak during the first several years of a person's life. (See the section "Underlying Mechanisms of Alcohol-Induced Damage to the Fetus" in the chapter on prenatal exposure to alcohol for additional information.)

Alcohol Inhibition of NMDA Receptor Function. In contrast to its effects on $GABA_A$ receptors, alcohol has been demonstrated to inhibit NMDA receptors (Fink and Gothert 1990; Lovinger et al. 1989; Popp et al. 1998; Simson et al. 1993; Wong et al. 1997; Woodward and Gonzales 1990). Some areas in the brain contain NMDA receptors that show a reduced sensitivity to the inhibitory effects of alcohol, suggesting that different regions of the brain may use different receptor subunits to assemble mature NMDA receptors. Another possibility is that, as with the $GABA_A$ receptor, intracellular activities such as phosphorylation may render NMDA receptors more or less sensitive to alcohol. Recent research suggests that both of these processes are likely to be involved in mediating alcohol's effects on the brain.

For example, several studies using cloned NMDA receptors expressed in *Xenopus* oocytes indicate that NR1/2A- and NR1/2B-containing NMDA receptors are more sensitive to alcohol than those expressing NR1/2C or NR1/2D subunits (Buller et al. 1995; Chu et al. 1995; Masood et al. 1994; Mirshahi and Woodward 1995). Not all investigators have reported this finding, however, which suggests that other factors may influence the sensitivity of the receptor to alcohol (Kuner et al. 1993). One group found that NR1/2A receptors that were made calcium impermeable by changing a single amino acid in the TMII pore region were less sensitive to alcohol than the wild-type receptor was (Mirshahi and Woodward 1995). Further investigation revealed that NR1/2A receptors were more sensitive to alcohol

when the amount of calcium that entered the oocyte was increased. A small portion of a piece of the NR1 subunit lying inside the cell mediates this calcium-dependent enhancement of the alcohol inhibition (Mirshahi et al. 1998).

Findings from animal studies are consistent with the results obtained using recombinant receptors expressed in a cell line (HEK 293 cells) that NMDA receptors with the NR1/2B combination are more sensitive to inhibition by alcohol than other NMDA receptors are (Blevins et al. 1997; Lovinger 1995; Yang et al. 1996). The magnitude of alcohol's inhibition was sometimes less than that seen when these same receptors were expressed in *Xenopus* oocytes. These results suggest that factors such as phosphorylation or receptor clustering may influence the sensitivity of NMDA receptors to alcohol. Several research groups are actively investigating these factors, which may help explain why NMDA receptors in some brain areas are more sensitive to alcohol than others.

Previous research has shown that the alcohol sensitivity of NMDA receptors in some cerebellar neurons is modulated by PKC (Snell et al. 1994). (The cerebellum is a brain structure primarily involved in balance and motor coordination.) More recent reports suggest that tyrosine kinases (enzymes that phosphorylate the amino acid tyrosine) may also be important in determining the alcohol sensitivity of NMDA receptors. As discussed in a previous section in this chapter ("From Cell Membrane to Nucleus: The Effects of Alcohol on Brain Neurons"), scientists have used a mouse line in which the gene for a specific tyrosine kinase, Fyn tyrosine kinase, was deleted from the mouse's genome (Miyakawa et al. 1997). These mice were more sensitive than normal mice to the sedative actions of alcohol. In addition, NMDA responses in hippocampal slices taken from Fyn tyrosine kinase-deficient mice did not show tolerance to the effects of sustained (15 minutes) alcohol exposure, as those from wild-type mice did. (The hippocampus is a brain structure involved in the consolidation of new memories.) The adaptation of NMDA responses to the inhibitory effects of alcohol (rapid tolerance) observed previously (Grover et al. 1994) may be due to changes in the phosphorylation of neuronal proteins such as NMDA receptors or proteins that interact with the receptor.

Despite these new developments showing that the NMDA receptor's alcohol sensitivity may be modulated by both subunit composition and phosphorylation, it is still not clear how alcohol actually inhibits the receptor. The NMDA receptor has many modulatory sites that control its function, and each one of these is a potential site of action for alcohol. However, to date there is no overwhelming evidence to indicate that alcohol interacts specifically with any of these modulatory sites (Chu et al. 1995; Masood et al. 1994; Mirshahi and Woodward 1995). Analysis of the behavior of single NMDA channels in the presence of alcohol revealed that alcohol did not alter the basic biophysical properties of the channel (channel conductance) or block the open channel (Wright et al. 1996). However, alcohol did reduce the number of times an individual channel would open; exposure to alcohol also decreased the amount of time the channel was open once it was activated. Thus, as with other channel types, these results suggest that alcohol acts at a site or sites on the NMDA receptor that influence the ability of the neurotransmitter to activate or control channel opening. The location of this site has not been identified. The recent findings of a binding site for alcohol on $GABA_A$ and glycine receptor subunits (Mihic et al. 1997) will greatly accelerate the search for a similar site on the NMDA receptor.

Alcohol Inhibition of AMPA/Kainate Receptor Function. Research shows that alcohol also inhibits ion flux through AMPA/kainate receptors. In cultured neurons and preparations of isolated spinal cord, NMDA receptors appear to be more sensitive to alcohol than AMPA/kainate receptors are, while there is less difference in sensitivity in recombinant expression systems than in cultured neurons (Dildy-Mayfield and Harris 1992; Lovinger et al. 1989). Not all neuronal AMPA/kainate receptors display a low alcohol sensitivity, however (Martin et al. 1995). These

findings suggest that factors such as phosphorylation or receptor-receptor communication, which can vary according to the type of experimental preparations used, may influence the alcohol sensitivity of AMPA/kainate receptors in neurons.

Studies by one group showed that the alcohol sensitivity of various GluR AMPA/kainate receptor subunits expressed in *Xenopus* oocytes could be altered by manipulations that increased or decreased intracellular levels of calcium (Dildy-Mayfield and Harris 1995). Alcohol's ability to inhibit the transmission of electrical signals was greatest, for example, in cell preparations containing the highest concentrations of calcium. This potentiation of the alcohol effect was prevented by an inhibitor of PKC, suggesting a role for both calcium and phosphorylation. It is not known whether manipulations that increase calcium would enhance the alcohol sensitivity of AMPA/kainate receptors expressed in neurons. If so, however, these receptors could be inhibited by alcohol during periods of intense neuronal firing when calcium levels inside the neuron are higher.

The results of many studies suggest that alcohol inhibits the two major classes of glutamate-activated ion channels—NMDA and AMPA/kainate—in the brain and spinal cord. Because these channels mediate both rapid and prolonged synaptic signaling, inhibition of these responses may underlie some of the intoxicating and sedative, anesthetic effects of alcohol. Defining the precise structural and physiologic requirements that control the alcohol sensitivity of these important ion channels may lead to the discovery of alcohol-sensitive and alcohol-insensitive forms of these receptors. Unequal distribution of these receptors across brain areas and among individuals could explain the differences in alcohol sensitivity that have been observed in both experimental cell systems and humans.

Nicotinic Receptors. The neurotransmitter acetylcholine activates a multisubunit class of ligand-gated ion channels known as nicotinic receptors. These receptors contribute to neuronal excitability and are closely related to those found in skeletal muscle. The primary function of most nicotinic receptors is to control, or gate, the flux of sodium ions across the neuronal membrane; some members of this family of receptors (the $\alpha 7$ receptors) are also permeable to calcium ions. Previous studies have shown that low concentrations of alcohol (10 micromolar) actually potentiate the effects of acetylcholine on muscle-type nicotinic receptors. As with the $GABA_A$ and glycine receptors, to which the nicotinic receptors are related, these effects are more pronounced at low concentrations of acetylcholine. Such findings suggest that alcohol may affect all three receptor types similarly, perhaps through common sites of action. The physiologic significance of these effects is unknown but warrants further investigation.

Results of recent investigations of the effect of alcohol on nicotinic receptors in cultured cells and on recombinant nicotinic receptors have yielded sometimes conflicting results. Alcohol was found to potentiate or inhibit these ion channels, depending on such factors as the concentration of acetylcholine, the duration of exposure to alcohol, and the concentration of alcohol (Covernton and Connolly 1997; Nagata et al. 1996; Yu et al. 1996).

In a recent study, alcohol reduced the firing of neurons in the cerebellum; this effect was modulated by compounds that acted on nicotinic receptors (Freund and Palmer 1997*b*). These findings suggest that the effects of alcohol on neuronal activity most likely involve multiple subtypes of neurotransmitter receptors, and that the activity of one ion channel can influence the sensitivity of other channels.

Serotonin Receptors. Serotonin (5-HT), an important neurotransmitter in the brain, activates a large family of 5-HT receptors. One member of this family is the $5\text{-}HT_3$ receptor, which is a ligand-gated ion channel that controls the flux of sodium ions into neurons. The $5\text{-}HT_3$ receptor is structurally similar to nicotinic receptors and is sensitive to alcohol. This receptor is expressed in discrete areas of the brain, including the limbic areas of the forebrain (which are involved in the expression of emotional behavior) and hindbrain structures that mediate nausea and vomiting.

These receptors appear to be localized to presynaptic regions of neurons, including axons and axon terminals. This distribution suggests that the 5-HT_3 receptor modulates the regulation of the release of neurotransmitters such as dopamine, acetylcholine, and serotonin. (See the section "Genetic Studies of Alcohol's Actions on the Brain" later in this chapter for further information on 5-HT receptors and alcohol's effect on these receptors.)

As demonstrated with glycine and $GABA_A$ receptors, alcohol potentiates the activity of 5-HT_3 receptors in neurons (Lovinger and White 1991) and in transfected cells (cells in which recombinant genes have been introduced) and *Xenopus* oocytes (Lovinger and Zhou 1994; Machu and Harris 1994). Alcohol appears to affect 5-HT_3 activity through interactions with the 5-HT_3 receptor that result in a more stable, open channel (Zhou and Lovinger 1996). Because of the similarity in amino acid sequence between 5-HT_3 and $GABA_A$ or glycine receptors, researchers have hypothesized that a site of action within the TMII domain of the 5-HT_3 receptor may mediate these effects.

Alcohol's potentiating effects on 5-HT_3 receptor function (discussed in more detail below) may underlie some of the observed increases in dopamine in limbic areas of the forebrain following alcohol consumption or exposure. These increases are thought to be crucial in mediating the rewarding effects of alcohol and many other drugs of abuse.

Voltage-Gated Ion Channels

Unlike ligand-gated ion channels, voltage-gated ion channels are activated when the membrane potential of the neuron is altered. The coordinated activity of voltage-gated sodium and potassium channels underlies the generation and propagation of action potentials along axons. Judging from their structural and genetic similarities, these channels appear to belong to a large family. Voltage-gated calcium channels respond to depolarization of the membrane and allow calcium to enter the neuron. This calcium can trigger the release of neurotransmitter from presynaptic terminals, thereby initiating synaptic signaling. Voltage-gated potassium channels function to repolarize the neuronal membrane after depolarization by gating the flux of positively charged potassium ions out of the neuron. Thus, these voltage-gated channels are crucial for neuronal activity and have been extensively examined for their alcohol sensitivity.

Previous research indicated that voltage-gated sodium channels were particularly insensitive to alcohol at concentrations that are associated with the behavioral effects of alcohol (10 to 100 mM). Voltage-gated calcium channels appeared to be relatively insensitive to the acute actions of alcohol and thus were not thought to be an important target for alcohol sensitivity. However, more recent evidence (discussed below) indicates that some calcium and potassium channels are sensitive to low concentrations of alcohol and thus may contribute to the depressant effects of alcohol on neuronal function.

Calcium Channels. Like other ion channels, voltage-gated calcium channels are subdivided into several classes (T, L, N, P, Q, and others) according to structural and pharmacologic properties. These channels are found on different parts of the neuron, where they provide a pathway for calcium flux during depolarization of the membrane.

Because of the heterogeneous distribution of these channel types on neurons, it has been difficult to accurately assess the effects of alcohol on individual calcium channel subtypes. Scientists use strategies to block or encourage the activity of specific channel subtypes in order to isolate them for observation of alcohol's effects. With use of these manipulations, previous studies using intact neurons showed that alcohol inhibits some calcium channel receptor subtypes. Some subtypes (L and N) were somewhat sensitive to alcohol, whereas others (T and P) appeared to be relatively insensitive to alcohol (Hall et al. 1994; Twombly et al. 1990). More recent studies in a cell line (PC-12) that shares characteristics with neurons also suggest differences in sensitivities of the various subtypes of receptors. In recent

experiments in these cells, incubation with alcohol inhibited the activity of certain channel subtypes (N and P/Q), but only after several minutes (Solem et al. 1997). The effect was not observed in cells treated with an activator of the enzyme protein kinase A or by an inhibitor of a phosphatase (a phosphate-removing enzyme). These results suggest that phosphorylation of a cellular protein may be critical to alcohol's effects on calcium ion channels, although additional research is needed to confirm the extent to which observations in this cell line parallel what occurs in neurons.

Potassium Channels. A wide array of potassium channels have been cloned and grouped into several classes based upon their voltage dependence and sensitivity to intracellular ligands such as calcium and adenosine triphosphate (ATP, a molecule important in the energy metabolism of cells).

Several potassium channels have been found to be relatively insensitive to alcohol concentrations below 100 mM. Scientists have shown that alcohol inhibits one class of these receptors—Shaw2 channels (Covarrubias and Rubin 1993; Covarrubias et al. 1995). Analysis of the inhibitory effect of alcohol on the activity of single channels of this type indicated that alcohol reduced the probability that the channel would enter a long-duration open state. The investigators suggested that the site at which alcohol acted to cause this effect was on a section of the ion channel thought to be involved in controlling the opening or gating of the ion pore of the channels. Results of experiments in which the effects of single-amino acid substitutions at the site were observed suggest that the amino acid sequence—and changes involving more than a single amino acid—played a role in determining alcohol sensitivity. The amino acid sequence may determine the size of a pocket with which alcohol interacts on the channel protein. Results of another study also suggested the existence of a pocket on these channels that mediates alcohol's actions. The cloned channels were studied by expressing them in *Xenopus* oocytes and observing the effects of alcohols with more than eight carbons. The results suggested that the actions of alcohols on these channels were channel specific and therefore due to differences in the amino acid sequence (Chu and Treistman 1997).

Other studies have shown that processes other than the ion channel itself may be the targets for alcohol. Calcium-activated potassium channels are important in regulating neuronal excitability and are activated by increases in intracellular calcium that arise during depolarization of the neuronal membrane. A recent study found that alcohol increases the activity of the BK type of channel in concentrations between 10 and 100 mM (Dopico et al. 1996). This increased activity was manifested by an increase in the probability that the channel would be in an open, conducting state. Although the onset of the alcohol effect was observed as soon as the alcohol was added to the preparation, recovery of the effect upon washout of the alcohol required several minutes, suggesting that processes other than the channel itself may be involved in alcohol's effect. Similar results emerged in another study of alcohol and BK channels; however, the alcohol effect required several seconds to become apparent and its effects reversed after approximately 30 seconds of exposure (Jakab et al. 1997). In addition, the effects of alcohol were blocked by inhibitors of PKC at lower levels of alcohol. Inhibitors of cellular phosphatases enhanced the potentiating effects of alcohol on BK channel activity, suggesting that alcohol-induced phosphorylation of the channel or related proteins underlies this effect.

Alcohol and Neurotransmitter Systems

The research described above clearly shows that alcohol exerts many of its neurobehavioral effects via its direct and indirect modulation of ion channels. Alcohol's opposite effects on excitatory glutaminergic receptors and inhibitory $GABA_A$ and glycine receptors appear to be largely responsible for its intoxicating and sedative effects. However, because alcohol is considered an addictive substance, there is great interest in defining the molecular and cellular sites of action that underlie its addictive potential. A number of studies in this area have focused on the

interaction between alcohol and the neurotransmitters dopamine and 5-HT, both of which have been implicated in the reinforcing properties of alcohol and other drugs of abuse.

Biochemical Studies

A basic hypothesis in the field of addiction biology is that addictive drugs lead to increases in release of the neurotransmitter dopamine, which plays a role in motivation and reinforcement. The increase in dopamine is observed in a specific part of the brain called the nucleus accumbens, which is thought to play a key role in the rewarding or reinforcing effects of alcohol (Imperato and Di Chiara 1986; Wozniak et al. 1991). Several techniques have been developed and used to study the actions of alcohol on dopamine release, including electrophysiology, microdialysis, and in vivo voltammetry.

The basis for these changes in extracellular dopamine is unclear. The changes may result from alcohol's direct effect on the release of dopamine or from alcohol's ability to enhance dopamine release indirectly through effects on other proteins such as ion channels. An example of indirect enhancement is alcohol's potentiation of presynaptic 5-HT_3 receptors, which have been shown to contribute to depolarization-induced neurotransmitter release.

Several studies have examined both direct and indirect effects of alcohol on dopamine release. Research has shown, for example, that a high concentration of alcohol delivered through a microdialysis probe increased dopamine levels in the ventral tegmental area of the brain (Yan et al. 1996). (The ventral tegmental area is an area where dopaminergic fibers originate; these fibers project to forebrain areas thought to be involved in mediating the sensation of reward.) This release of dopamine was not attenuated by the use of a calcium-free solution, indicating that the increase in dopamine levels did *not* result from the presynaptic, calcium-dependent process known as exocytosis, through which neurotransmitters are released. Another study demonstrated that alcohol-induced increases in extracellular levels of dopamine in the striatum, a brain area highly enriched in dopaminergic terminals, occurred only at high concentrations of alcohol (more than 170 mM in the dialysis probe) (Yim et al. 1997). The researchers concluded that when alcohol was given locally through the probe, concentrations of alcohol associated with intoxication had no effect on extracellular levels of dopamine.

These findings have been corroborated by studies using a different technique, in vivo voltammetry, which measures the presence of extracellular dopamine electrochemically. In these studies, alcohol administered locally via microinjection did not alter basal (pretreatment), unstimulated levels of dopamine in the nucleus accumbens (Samson et al. 1997) or the striatum (Lin and Chai 1995; Wang et al. 1997). Despite this lack of response, alcohol did slow the clearance of both endogenous and exogenous dopamine from the extracellular fluid, suggesting that alcohol may affect the dopamine transporter molecule, which functions to clear the synapse of dopamine following release. During periods of neuronal firing and release of dopamine, alcohol thus may directly prolong the actions of dopamine at postsynaptic receptors.

Evidence for a 5-HT_3-mediated mechanism of alcohol's action on dopamine levels has also been described. One study showed that drugs that selectively activate the 5-HT_3 receptor increased extracellular levels of dopamine as measured by in vivo microdialysis (Campbell et al. 1996). Dopamine levels were also enhanced when animals were given alcohol intraperitoneally (into the abdominal cavity); this increase was blocked by a selective antagonist of 5-HT_3. These results suggest that the effects of ingested alcohol on dopamine release may be mediated via enhancement of 5-HT_3 receptors located on dopaminergic nerve terminals.

A series of studies by Brodie and co-workers demonstrated that alcohol increased the firing rate of neurons in the ventral tegmental area and that the effects of alcohol were augmented in the presence of 5-HT and 5-HT agonists (substances

that stimulate the activity of 5-HT) (Brodie et al. 1990, 1995). Whether these results were due to an effect on 5-HT_3 receptors or on another mechanism could not be determined. However, these data are consistent with the idea that alcohol can alter a cell's firing rate and the release of dopamine in an area of the brain associated with reward and reinforcement.

Neurobehavioral Studies

Following the observation that alcohol potentiated 5-HT_3 electrical activity in isolated neurons, a large number of neurobehavioral studies were performed to examine the involvement of this and other 5-HT receptor subtypes in mediating alcohol's actions on the brain. Many of these studies used the technique of drug discrimination to probe the neural sites of action of alcohol. (See the section "Neurobiological and Neurobehavioral Mechanisms of Chronic Alcohol Drinking" later in this chapter and the box "Animal Models for Alcoholism," which detail the different types of tests and animal models used to study the impact of alcohol exposure on behavior.)

Drug discrimination procedures involve training an animal to recognize the effects or rewards of a particular drug, then testing other drugs to determine whether they can substitute for the training drug. The 5-HT_3 antagonists generally block the discriminative stimulus effects—the effects that enable an animal to distinguish alcohol from other substances—of alcohol, although there is considerable variability in this respect among these agents (see review by Grant 1995). The effects of these drugs on alcohol absorption and on other receptor subtypes complicate interpretation of these tests. The development of more selective agents would help to unravel the role of the 5-HT_3 receptor in mediating the neurobehavioral effects of alcohol.

Recent behavioral studies have revealed that some 5-HT receptors that are G protein coupled—linked to intracellular signaling processes—may be involved in mediating alcohol's effects. In one study, agonists with selectivity at 5-HT_{1B} but not 5-HT_{1A} receptors produced effects that were similar to alcohol in drug discrimination tests (Grant et al. 1997). Interestingly, these effects depended on the training dose of alcohol and were most marked at lower alcohol concentrations. These results are consistent with in vitro findings that show differences in the sensitivity of various receptors to alcohol.

The involvement of the 5-HT_{1B} receptor in mediating some of the effects of alcohol has also been suggested by research on a mouse strain that lacks this receptor subtype. These mice had a twofold elevation in their daily alcohol consumption, compared with wild-type mice, in the presence of normal food and water intakes (Crabbe et al. 1996). (A subsequent study did not replicate this result in knockout mice [Crabbe et al. 1999].) The mutant mice were also less sensitive to the motor-incoordinating effects of alcohol and developed tolerance to these effects at a slower rate than wild-type animals did. Subsequent neurobehavioral studies using these knockout mice showed that 5-HT_{1B} knockout animals had a lower sensitivity to the rewarding effects of alcohol but no change in alcohol's aversive effects (Risinger et al. 1996). Taken together, these results suggest that the 5-HT_{1B} receptor is involved in both alcohol intake and reward, perhaps through a common mechanism. Although this underlying neurochemical mechanism has not yet been identified, researchers have hypothesized that this reduced sensitivity to alcohol's rewarding effects in these animals may result in higher levels of alcohol drinking. Whether these differences are related or due to differences in the effects of alcohol on firing rate and extracellular dopamine levels in the mesolimbic areas of the brains of these mice is not known. (See also the discussion of 5-HT_{1B} knockouts in the section "Genetic Studies of Alcohol's Action on the Brain" later in this chapter.)

In Closing

Both ligand-gated and voltage-sensitive ion channels are important targets for alcohol in the brain. The distribution of many of these channels in synapses and the channels' critical involvement in regulating neuronal excitability make their alcohol sensitivity especially important with

respect to alcohol's behavioral effects. Several conclusions can be reached with respect to the findings of the studies of alcohol sensitivity of various neuronal ion channel proteins.

First, clear and consistent evidence demonstrates that alcohol specifically and selectively alters the function of certain ion channels. In the case of the $GABA_A$ and glycine receptors, compelling evidence exists to suggest that alcohol's effects are mediated by interaction with certain amino acids on the receptor. Using this information, researchers should be able to verify the presence of a similar site of action on other related receptors, such as those activated by acetylcholine and 5-HT, which are similar in composition to $GABA_A$ and glycine receptors.

Elucidation of a primary site of action for alcohol on glutamate-activated ion channels and voltage-sensitive potassium channels is expected to be somewhat more difficult. NMDA and non-NMDA channels share a high degree of structural similarity, especially in their pore regions, but are only remotely related to $GABA_A$ and glycine receptors. Thus, it may be difficult to extrapolate findings directly from one major family of ion channels to another. However, as researchers learn more about the determinants of alcohol sensitivity among different channel types, various techniques, such as site-directed mutagenesis, which leads to a mutation in a specific gene or at a specific location along the DNA strand, can be used to identify the locations of alcohol-binding sites on glutamate and other receptors.

Second, the effects of alcohol on ion flux appear to be mediated not by simple blocking of the ion pore but by alterations in channel gating. Most, if not all, studies that have examined the effects of alcohol on the function of single-ion channels have found that alcohol alters the probability that the channel will be open. These findings suggest that the site that mediates alcohol's actions most likely is located close to those areas that control gating of the channel. Such research results and hypotheses place the focus of attention on the transmembrane domains that traverse the membrane and the amino acids that may serve to couple one transmembrane domain to another.

Finally, it is clear that alcohol does not need to act directly on the channel protein to alter its function. The effects of alcohol on voltage-sensitive calcium channels and perhaps BK channels may involve other cellular processes that are activated by alcohol. Once activated, these other processes can then profoundly alter the function of the ion channel via mechanisms such as phosphorylation or direct protein-protein interaction. One of the rapidly developing areas of neuroscience concerns the organization of ion channels in the neuronal membrane. It is clear that ion channels are not distributed randomly in the membrane, but are localized in clusters in association with other signaling and structural proteins. Researchers have described a whole family of receptor clustering proteins that appear to act as scaffolds that allow complementary receptors and cellular signaling proteins (such as kinases and phosphatases) to be located at the same site. Alcohol's effects on individual ion channels may thus be a sum of its direct action on ion channel gating and its influence on the regulation of ion channel activity by these closely associated processes.

Research on the neurotransmitters and receptors involved in the rewarding aspects of alcohol is providing insight into how alcohol influences the function of these systems and how the changes are manifested in the response to alcohol in an intact organism.

As the cellular and molecular processes involved in the interplay between alcohol and neurons are identified and understood, connections between these processes and the neurobehavioral effects of alcohol will be strengthened. Research, in turn, can then increasingly focus on the development of therapeutic agents that interfere with these processes.

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